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Repression of Antibiotic Production and Sporulation in *Streptomyces coelicolor* by Overexpression of a TetR Family Transcriptional Regulator▽†

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The overexpression of a regulatory gene of the TetR family (*SCO3201*) originating either from *Streptomyces lividans* or from *Streptomyces coelicolor* was shown to strongly repress antibiotic production (calcium-dependent antibiotic [CDA], undecylprodigiosin [RED], and actinorhodin [ACT]) of *S. coelicolor* and of the *ppk* mutant strain of *S. lividans*. Curiously, the overexpression of this gene also had a strong inhibitory effect on the sporulation process of *S. coelicolor* but not on that of *S. lividans*. SCO3201 was shown to negatively regulate its own transcription, and its DNA binding motif was found to overlap its −35 promoter sequence. The interruption of this gene in *S. lividans* or *S. coelicolor* did not lead to any obvious phenotypes, indicating that when overexpressed SCO3201 likely controls the expression of target genes of other TetR regulators involved in the regulation of the metabolic and morphological differentiation process in *S. coelicolor*. The direct and functional interaction of SCO3201 with the promoter region of *scbA*, a gene under the positive control of the TetR-like regulator, ScbR, was indeed demonstrated by *in vitro* as well as *in vivo* approaches.

The development of the Gram-positive genus *Streptomyces* is characterized by a complex morphological differentiation process thought to be triggered by conditions of nutritional limitation that often correlate with high cell density (41). This process includes the arising of an aerial mycelium from the vegetative mycelium and then the differentiation of the tips of the aerial hyphae into spores (10, 15). This process is coupled to a metabolic differentiation that correlates with the production of a wide range of pharmaceutically important secondary metabolites, including antibacterial, anticancer, and immunosuppressive drugs (8, 10). The genes responsible for the biosynthesis of these secondary metabolites are clustered in the genome and coordinately regulated by pathway-specific transcriptional regulators (1, 4, 18, 19, 62, 70). The expression of these specific regulators linked to the biosynthetic pathways is directly or indirectly controlled either by positive pleiotropic regulators, such as AfsR2 (69), AfsS (29), AtrA (65), PtpA (67), PkaD (68), and the two-component systems AfsK/AfsR (66) and EcrA1/EcrA2 (39), by negative regulators, including the two-component systems AbsA1/AbsA2 (44), PhoR/PhoP (55), and CutR/CutS (9), or by enzymatic systems, such as Ppk (16). The pleiotropic regulators, thought to sense a variety of extracellular or intracellular signals related to nutriment availability, cell crowding, or energy shortage, are necessary to trigger the necessary metabolic adjustments to adapt to these conditions (27, 43), whereas the *ppk* gene is thought to act as an ATP-regenerating enzyme (54).

Streptomyces coelicolor A3(2) is usually used as the reference strain to study morphological and metabolic differentiation in relation with antibiotic biosynthesis (10, 15). *S. coelicolor* has long been known to produce four major antibiotics, actinorhodin (ACT) (40), undecylprodigiosin (RED) (13), methylenomycin (MMY) (36), and calcium-dependent antibiotic (CDA) (21), and recently, two novel ones were characterized, CPK, a putative type I polyketide (17, 50), and albaflavenone, a sesquiterpene antibiotic (72). ACT is a secondary metabolite of the polyketide family that is strongly produced by *S. coelicolor* but only weakly produced by its close relative, *Streptomyces lividans*. However, *S. lividans* has the genetic capability to produce this compound since the weak production of ACT could be stimulated by various genetic manipulations that include the overexpression of the pathway-specific activator gene *act*II-ORF4 (7), the *afsR* and *afsR2* (now *afsS*) genes (35), the *rep* gene (42), and the phosphotyrosine protein phosphatase-encoding gene *ptpA* (67). The inactivation of other genes, such as *ppk* (11), or mutations in the *rpoB* gene (25) or in the ribosomal protein S12 (23) are also leading to an enhancement of ACT production, indicating that ACT production was subjected to complex positive and negative controls. Furthermore, the extracellular addition of the signal molecule *S*-adenosylmethionine (34) or of ATP (38) or the replacement of glucose by glycerol in a minimal medium (35) was also shown to enhance ACT production in *S. lividans*. However, a systemic understanding of the regulation of antibiotic biosynthesis and in particular of the molecular basis of the very different abilities of *S. lividans* and *S. coelicolor* to produce ACT is still lacking. In order to tackle this question, we hypothesized that a major negative regulator of ACT production might be

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present in *S. lividans* but absent or nonfunctional in *S. coelicolor*. In an attempt to get this putative negative regulator, a genomic library of *S. lividans* DNA was constructed into the high-copy-number replicative plasmid pIJ702 (33) and introduced into *S. coelicolor*. An unique white transformant (ACT^-) was obtained. This phenotype was shown to result from the overexpression of a regulator of the TetR family (equivalent to SCO3201 of *S. coelicolor*). The consequences of the overexpression and of the deletion of *SCO3201* on the metabolic and morphological differentiation processes of *S. coelicolor* and *S. lividans* (wild type [wt] and *ppk* mutant) were assessed. The ability of SCO3201 to negatively regulate its own transcription was demonstrated by both *in vitro* and *in vivo* approaches, and the sequence of its operator site located in its own promoter region was determined. Sequences related to the SCO3201 operator site were found in the promoter region of *scbA*, a gene encoding a butyrolactone-synthesizing enzyme (61). These sequences were shown to be part of the binding site of ScbR, a TetR family regulator, positively controlling *scbA* expression, as SCO3201 does when overexpressed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *S. coelicolor* M145 ($SCP1^-$ $SCP2^-$) (33), *S. lividans* TK24 (*str-6* $SLP2^-$ $SLP3^-$) (33), and its derived *ppk* mutant, the Δ*ppk*::Ω*hyg* strain (11), were used in this study. *Escherichia coli* strains DH5α and D41 were used for routine subcloning work and as a host for protein expression, respectively. For the biological assay of calcium-dependent antibiotic (CDA), Oxoid nutrient agar (ONA) medium (33) was used for cultivation of *Streptomyces* strains, and *Micrococcus luteus* DSM1790 was used as an indicator strain. *S. lividans* TK24 chromosomal DNA was isolated from HT medium (33)-grown cultures. SFM medium (33) was used for the assessment of the completion of the sporulation process as well as for spore collection. Protoplast generation, transformation, and regeneration on R2YE medium (33) were performed according to a previously documented procedure (26). Spores of various *Streptomyces* strains were spread on the surfaces of cellophane discs (from Cannings Packaging, Limited, United Kingdom) laid on the surface of R2YE agar medium with no phosphate supplementation (condition of phosphate limitation). When necessary, ampicillin, apramycin, and thiostrepton were included in the solid R2YE medium at a final concentration of 50 μg/μl, whereas thiostrepton was added at a concentration of 5 μg/μl in a liquid R2YE culture.

Construction of a genomic library of *S. lividans* TK24. Fragmented mycelium originating from a 36-h preculture of *S. lividans TK24* in 10 ml HT medium was used to inoculate 100 ml of HT medium in 500-ml flasks. The culture was incubated overnight at 30°C, and chromosomal DNA was extracted from the mycelium using the CTAB method described in *Practical Streptomyces Genetics* (33). Total DNA (5 μg) was partially digested with the addition of 30 units of Sau3AI (Fermentas), followed by incubation at 37°C for 5 min. The digestion products were separated on a 0.8% agarose gel in Tris-acetate-EDTA buffer. Approximately 3- to 5-kb-long DNA fragments were electroeluted from the gel, treated with phenol-chloroform–isoamyl alcohol, and precipitated. The recovered Sau3AI DNA fragments were ligated into the high-copy-number vector pIJ702 (32), digested with BglII, and dephosphorylated. Subsequently, the ligation mixture was used to transform *S. coelicolor* M145 protoplasts, and transformants were selected in the presence of thiostrepton (50-μg/ml final concentration) on R2YE medium.

Overexpression of $SCO3201_{SL/SC}$ in *Streptomyces*. The *S. lividans*/*S. coelicolor* *SCO3201* ($SCO3201_{SL/SC}$) gene fused to a six-histidine tag at its 3′ end (corresponding to the carboxy-terminal end of the protein) was retrieved as an XbaI-PstI fragment from pET21a$^+$-$SCO3201^{SL}$ and pET21a$^+$-$SCO3201^{SC}$, respectively (see below), and ligated into pWHM3-*ermE* cut by XbaI and PstI to yield pWHM3-$SCO3201^{SL}$ and pWHM3-$SCO3201^{SC}$, respectively. pWHM3-*ermE* is a derivative of pWHM3 with a 279-bp KpnI-BamHI *ermE* fragment containing the *ermE* promoter (5) as well as a plausible ribosomal binding site (GenBank accession no. M11200). Protoplasts of *S. coelicolor* M145 as well as the wild type and the *ppk* mutant of *S. lividans* were transformed by pWHM3-$SCO3201^{SL}$ and pWHM3-$SCO3201^{SC}$.

Determination of CDA, RED, and ACT production. In order to evaluate the CDA production, 2- by 2-cm patches made with 10^6 spores of the various strains were made on Oxoid nutrient agar (ONA) (33) and incubated at 30°C for 48 h. ONA plates were overlaid with an overnight culture of *Micrococcus luteus* in LB medium diluted 1/100 in soft nutrient agar (SNA) (33) with or without $Ca(NO_3)_2$ at 12 mM (final concentration). Plates were incubated at 37°C overnight. The diameters of the inhibition zones, formed only in the presence of $Ca(NO_3)_2$, were measured to estimate CDA production.

In order to quantify the production of RED (mostly intracellular) and ACT (both intra and extracellular), 10^7 spores of the various strains were plated on cellophane discs laid on the surface of R2YE solid medium (no P_i supplementation) and incubated at 30°C for 84 h. In order to assay the intracellular concentrations of RED and ACT, the mycelium corresponding to one-quarter of an 8.5-cm-diameter plate (approximately 40 mg of dry cell weight) was extracted by the addition of 1 ml of methanol and of 1 ml of KOH (1 N), respectively, and continuous vortexing for 30 min at 4°C. The resulting RED extract was acidified to pH 2 to 3 with the addition of 1 ml HCl (1 N), and the optical density at 530 nm (OD_{530}) was measured with a spectrophotometer (Beckman) against a blank constituted by methanol and 1 N HCl (1:1 ratio in volume). The OD_{640} of the resulting ACT extract was measured with the same spectrophotometer against a blank constituted by 1 N KOH (33). The mycelium corresponding to the remaining half of the plate was saved for dry-cell-weight (biomass) determination. In order to assay for extracellular ACT, a volume of R2YE agar medium corresponding to one-quarter of the individual plate was smashed and allowed to diffuse in 10 ml water for 1 h at 4°C. After centrifugation, supernatant was collected and 10 ml 1 N KOH was added and mixed by inversion. Subsequently, 10 ml HCl (3 N) was added. The resultant mixture was incubated on ice for 10 min. Precipitated ACT was collected by centrifugation at 13,000 rpm for 10 min. The resulting pellet was diluted in 1 ml of KOH (1 N), and the OD_{640} of the solution was determined. The assays for each antibiotic were completed in triplicate for each strain. The mean values ± standard deviations were calculated and are shown in Tables 2 to 5.

Construction of disrupted mutants of *SCO3201* in *S. coelicolor* M145 and in *S. lividans* TK24 (wild type and *ppk* mutant). In order to inactivate *SCO3201* in the wild-type and the *ppk* mutant strains of *S. lividans* TK24 as well as in *S. coelicolor* M145, two 1-kb DNA fragments flanking the *SCO3201* coding sequence were amplified by PCR from the chromosome DNA of *S. lividans* TK24 and *S. coelicolor* M145, using the two pairs of primers, one pair comprising UtetR-BamHI and UtetR-XbaI and one pair comprising DtetR-HindIII and DtetR-XbaI (Table 6). The resulting PCR fragments were digested with the corresponding restriction enzymes and ligated in the presence of an apramycin resistance cassette (recovered as an XbaI fragment from pHp45Ωapra [6]) into the thermosensitive pGM160 vector (47) cut by BamHI and HindIII. The resulting plasmids suitable for gene interruption [pGM160/$SCO3201^{SC/SL}$::*acc(3)IV*] were introduced into *S. coelicolor* M145 as well as into *S. lividans* TK24 (wild type and *ppk* mutant). After three rounds of sporulation at 37°C, the progeny was screened for the apramycin-resistant and thiostrepton-sensitive phenotype to yield the expected *SCO3201* disruptive mutants. The primer pair comprising ItetR1 and ItetR2 (Table 6) was used to confirm the replacement of the *SCO3201* gene by the *aac(3)IV* cassette in the three strains by PCR.

Purification of His-tagged $SCO3201_{SL/SC}$ in *E. coli*. In order to purify $SCO3201_{SL/SC}$ from *E. coli*, the *SCO3201* coding genes of *S. coelicolor* M145 ($SCO3201_{SC}$) and *S. lividans* TK24 ($SCO3201_{SL}$) were amplified by PCR from the genomic DNA of each strain, using the primers ExpTetR1-NdeI and ExpTetR2-XhoI (Table 6). The PCR products were cloned into pET21a$^+$ cut by NdeI and XhoI to yield pET21a$^+$-$SCO3201^{SC}$ and pET21a$^+$-$SCO3201^{SL}$, in which the transcription of the cloned gene is under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lac* promoter (58). The two plasmids were transformed into *E. coli* D41, a convenient host for protein expression (46). The resulting transformants were cultivated in LB medium at 37°C to give an OD_{600} of 0.6. IPTG was added at a final concentration of 1 mM for induction, and incubation was pursued for 4 extra hours. The cells were collected by centrifugation, resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole), and disrupted by sonication. The cell debris was removed by centrifugation at 12,000 rpm at 4°C for 15 min. The resulting cell extract was passed through HIS-Select nickel affinity Gel (P6611; Sigma), and six-histidine-tagged SCO3201 (His_6-SCO3201) was purified to near-homogeneity in accordance with the protocol of the manufacturer.

Preparation of *Streptomyces* cell crude extracts. Two hundred milligrams of mycelia of 24-h-grown cultures from each individual strain was collected from lawns grown on R2YE solid medium and washed with ice-cold water containing 5 mM EDTA. The mycelium was suspended in 2 ml sonication buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol [DTT], 10% glycerol, and

TABLE 1. Strains and plasmids used in this study

Strain and plasmid	Description	Reference or source
Strains		
S. coelicolor		
M145	Prototroph; SCP1$^-$ SCP2$^-$	33
M145/pWHM3	*S. coelicolor* M145 carrying pWHM3-*ermE*	This study
M145/pWHM3-*SCO3201*^{SC}	*S. coelicolor* M145 carrying pWHM3-*SCO3201*^{SC}	This study
M145/pWHM3-*SCO3201*^{SL}	*S. coelicolor* M145 carrying pWHM3-*SCO3201*^{SL}	This study
M145/Δ*SCO3201*^{SC}::*acc(3)IV*	*S. coelicolor* M145 with *SCO3201* replaced by the *acc(3)IV* cassette	This study
M145/pIJ4083-*SCO3201*$_p$	*S. coelicolor* M145 carrying pIJ4083-*SCO3201*p (promoter region of *SCO3201*)	This study
M145/Δ*SCO3201*^{SC}::*acc(3)IV*/pIJ4083-*SCO3201*$_p$	*S. coelicolor* M145/Δ*SCO3201*^{SC}::*acc(3)IV* carrying pIJ4083-*SCO3201*p	This study
S. lividans		
TK24	*str-6* SLP2$^-$ SLP3$^-$	33
TK24/pWHM3	*S. lividans* TK24 carrying pWHM3-*ermE*	This study
TK24/pWHM3-*SCO3201*^{SC}	*S. lividans* TK24 carrying pWHM3-*SCO3201*^{SC}	This study
TK24/pWHM3-*SCO3201*^{SL}	*S. lividans* TK24 carrying pWHM3-*SCO3201*^{SL}	This study
TK24/Δ*SCO3201*^{SL}::*acc(3)IV*	*S. lividans* TK24 with *SCO3201* replaced by the *acc(3)IV* cassette	This study
TK24/pIJ4083-*SCO3201*$_p$	*S. lividans* TK24 carrying pIJ4083-*SCO3201*p	This study
TK24/Δ*SCO3201*^{SC}::*acc(3)IV*/*pIJ4083-SCO3201*$_p$	*S. lividans* TK24/Δ*SCO3201*^{SL} carrying pIJ4083-*SCO3201*p	This study
TK24/Δ*ppk*::Ω*hyg*	*S. lividans* TK24 with the *ppk* gene disrupted by the Ω*hyg* cassette	11
TK24/Δ*ppk*::Ω*hyg*/pWHM3	*S. lividans* TK24/Δ*ppk* carrying pWHM3-*ermE*	This study
TK24/Δ*ppk*::Ω*hyg*/pWHM3-*SCO3201*^{SC}	*S. lividans* TK24/Δ*ppk* carrying pWHM3-*SCO3201*^{SC}	This study
TK24/Δ*ppk*::Ω*hyg*/pWHM3-*SCO3201*^{SL}	*S. lividans* TK24/Δ*ppk* carrying pWHM3-*SCO3201*^{SL}	This study
TK24/Δ*ppk*::Ω*hyg*/Δ*SCO3201*^{SL}::*acc(3)IV*	*S. lividans* TK24/Δ*ppk*::Ω*hyg* with *SCO3201* replaced by the *acc(3)IV* cassette	This study
TK24/Δ*ppk*::Ω*hyg*/pIJ4083-*SCO3201*$_p$	*S. lividans* TK24/Δ*ppk*::Ω*hyg* carrying pIJ4083-*SCO3201*p	This study
TK24/Δ*ppk*::Ω*hyg*&Δ*SCO3201*::*acc(3)IV*/pIJ4083-*SCO3201*$_p$	*S. lividans* TK24/Δ*ppk*::Ω*hyg*-Δ*SCO3201*::*acc(3)IV* carrying pIJ4083-*SCO3201*p	This study
E. coli		
DH5α	F *recA lacZ*M15	Promega
C41 (DE3)	Mutant of DH5α; overcomes toxic effects associated with overexpression	46
ET12567	*dam dcm hsdS*	33
Plasmids		
pIJ702	High-copy-no. *Streptomyces* replicative plasmid	33
pIJ702-*SCO3200*&*SCO3201*	pIJ702 carrying the complete *SCO3200* and *SCO3201* genes	This study
pIJ4083	High-copy-no. *Streptomyces* replicative promoter probe vector with *xylE* as the reporter gene; Tsr^r	33
pIJ4083-*SCO3201*p	pIJ4083 carrying the promoter region of *SCO3201* cloned upstream of the *xylE* reporter gene	This study
pWHM3-*ermE*	High-copy-no. *E. coli-Streptomyces* shuttle expression vector carrying the *ermE* promoter; Tsr^r Amp^r	5
pWHM3-*SCO3201*^{SC}	pWHM3-*ermE* carrying the *SCO3201*$_{SC}$ gene cloned downstream of the *ermE* promoter; Tsr^r Amp^r	This study
pWHM3-*SCO3201*^{SL}	pWHM3-*ermE* carrying the *SCO3201*$_{SL}$ gene cloned downstream of the *ermE* promoter; Tsr^r Amp^r	This study
pGM160	High-copy-no. *E. coli-Streptomyces* shuttle replicative vector; temp-sensitive replication in *Streptomyces*; Amp^r Tsr^r	47
pGM160-*SCO3201-acc(3)IV*	pGM160 carrying the apramycin resistance gene cassette *acc(3)IV* flanked by the regions (1 kb) located upstream and downstream of *SCO3201*	This study
pET21a$^+$	*E. coli* overexpression vector; Amp^r	Novagen
pET21a$^+$-*SCO3201*^{SC}	C-terminal His$_6$ fusion of SCO3201$_{SC}$ cloned into pET21	This study
pET21a$^+$-*SCO3201*^{SL}	C-terminal His$_6$ fusion of SCO3201$_{SL}$ cloned into pET21	This study

TABLE 2. Quantitative analysis of calcium-dependent antibiotic

Strain	Diam of inhibition zone (cm)[a]			
	pWHM3	pWHM3-*SCO3201*	WT	Δ*SCO3201*
S. coelicolor M145	6.07 ± 0.21	ND	6.20 ± 0.17	6.27 ± 0.25
S. lividans TK24	3.97 ± 0.06	ND	4.17 ± 0.29	4.20 ± 0.17
S. lividans TK24/Δ*ppk*::Ω*hyg*	3.80 ± 0.10	ND	3.93 ± 0.06	3.70 ± 0.20

[a] WT, wild type; ND, not detected.

1 mM phenylmethanesulfonyl fluoride). The mixture was then subjected to sonication to obtain a homogeneous solution, and cell debris was removed by centrifugation. The protein concentration of each sonicate was determined using the Bradford reagent (catalogue no. 500-0006; Bio-Rad). The protein concentrations of all samples were adjusted to 10 μg/μl before utilization for the electrophoretic mobility shift assay (EMSA).

EMSA. The 5′-biotin-labeled *SCO3201* promoter region was obtained by PCR based on the chromosome DNA of *S. coelicolor* M145, using primers PtetR2 (5′ biotin labeled; Eurogentec) and PtetR2′ (Table 6). The *SCO3201* promoter region (*SCO3201*p; 0.1 pmol) was incubated with purified His_6-SCO3201 or cell crude extracts from individual strains for 15 min at room temperature in a buffer containing 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1 mM EDTA, 1 mM DTT and 1 μg poly(dI-dC) in a total volume of 20 μl. After incubation, complexes and free DNA were resolved on a native polyacrylamide (5%) gel prerun at 100 V for 30 min with a running buffer containing 45 mM Tris-HCl (pH 8.3), 45 mM boric acid, and 10 mM EDTA and then run for 40 min at 100 V. Subsequently, the complexes and free DNA were transferred to a positively charged membrane (catalogue no. 130291; Appligene) using an electrophoretic transfer cell. After cross-linking under UV light at 254 nm for 10 min, visualization of the DNA signal from membrane was carried out using a LightShift chemiluminescence EMSA kit (catalogue no. 20148; Pierce, Rockford, IL).

The promoter regions of *scbA*, *scbR*, and *cpkO* were amplified by PCR from *S. coelicolor* genomic DNA, using primer pairs PscbA1/PscbA2, PscbR1/PscbR2, and PcpkO1/PcpkO2 (Table 6). The resulted DNA fragments were purified by a NucleoSpin Extract II column (catalogue no. 740 609.50; Macherey-Nagel). Fragment labeling was performed by PCR as described by Tiffert et al. (64), using the 5′-AGCCAGTGGCGATAAG-3′ primer that was labeled at its 5′ end with Cy5. These labeled fragments were used for EMSA. The DNA bands were visualized by fluorescence imaging using a Typhoon Trio+ variable-mode imager.

DNase I footprinting. DNase I footprinting assays with the *SCO3201* promoter region were performed using the fluorescence labeling procedure described by Yindeeyoungyeon and Schell (71). DNA probes for both coding and complementary strands were amplified by PCR from the genomic DNA of *S. coelicolor* M145 using the primers ItetR1 and GSP3 (Table 6), which were fluorescein labeled at the 5′ end of the coding and complementary strands, respectively. The resultant PCR products were purified from agarose gel (catalogue no. 740 609.50; Macherey-Nagel). The DNA probe (0.3 pmol) was incubated with 0.8 pmol of purified SCO3201 under the same conditions as those described above for EMSA, except that the reaction scale was enlarged to 50 μl. After the binding reaction, 5 μl of 5 mM $CaCl_2$ was added to each reaction mixture and DNase I (catalogue no. AM2222; Ambion) digestion was carried out with 2 μl of the 1:1,000 dilution (4×10^{-3} units) at 37°C for 1 min. The reaction was stopped with the addition of EDTA at a final concentration of 5 mM, and the reaction mixture was subsequently heated at 75°C for 10 min to totally inactivate DNase I. DNA was then purified with a NucleoSpin Extract II column (catalogue no. 740 609.50; Macherey-Nagel). After ethanol precipitation, the samples were resuspended in 30 μl of SLS buffer (Beckman Coulter) and subjected to capillary electrophoresis by loading into a CEQ 8000 sequencing apparatus (Beckman Coulter). The elucidation of the DNA sequence of the protected region was carried out by comigration of the footprint assay, the molecular standard (catalogue no. 608095; Beckman Coulter), and the corresponding sequencing reaction (catalogue no. 608120; Beckman Coulter).

5′ rapid-amplification-of-cDNA-ends (5′-RACE) PCR. In order to determine the transcriptional start site of *SCO3201*, total RNA was extracted from a 24-h-old culture of *S. coelicolor* M145 grown on solid R2YE medium using the NucleoSpin RNAII isolation kit (catalogue no. 740 955.50; Macherey-Nagel). Five micrograms of total RNA was used for reverse transcription with 2 pmol of a gene-specific primer, GSP1 (Table 6), using Superscript III first-strand synthesis supermix (catalogue no. 18080-400; Invitrogen). The sample was subsequently purified with a NucleoSpin Extract II column (catalogue no. 740 609.50; Macherey-Nagel). A homopolymeric A tail was added to the 3′ end of cDNA using terminal deoxynucleotidyl transferase (catalogue no. EP0161; Fermentas) in accordance with the manufacturer's instructions, and the sample was again purified with a NucleoSpin column. The poly(dA)-tailed cDNA was then used as a template for the PCR amplification using a poly(dT) primer (70 homopolymeric T) and a second inner-gene-specific primer, GSP2 (Table 6). An additional round of PCR was subsequently performed with a 1,000-fold dilution of the original PCR product as a template, using the poly(dT) primer and a nested GSP3 primer (Table 6), to obtain a single specific band. The final PCR products were sequenced using the nested GSP3 primer and the reaction kit (catalogue no. 608120; Beckman Coulter) according to the manufacturer's protocol on a CEQ8000 (Beckman Coulter) apparatus. The transcriptional start site was identified as the nucleotide immediately preceding the stretch of T residues complementary to the A tail in the sequence of the PCR product.

SCO3201 promoter probing using the promoter-less *xylE* gene encoding a catechol 2,3-dioxygenase. The *SCO3201* promoter region was amplified by PCR from the *S. coelicolor* M145 chromosome using primers PtetR1-XbaI and PtetR1-HindIII (Table 6). The resulting PCR product was ligated into the promoter probe vector pIJ4083 (33) cut by XbaI and HindIII. The ligation mixture was used to transform *S. lividans* TK24, and the correct structure of the promoter probe construct isolated from these primary transformants was checked by restriction analysis. This plasmid was then transformed into the three strains of interest and their corresponding *SCO3201* knockout mutants. These six strains were cultivated in R2YE liquid medium, and the *xylE* activity was assayed in the cell extract as previously described (28). Total proteins were measured using the Bradford reagent (catalogue no. 500-0006; Bio-Rad) to calculate the specific enzymatic activity.

Isolation of total RNA and nonquantitative RT-PCR. *Streptomyces* mycelia were collected from R2YE agar plates, and total RNA was isolated using a NucleoSpin RNA II kit (catalogue no. 740955.50; Macherey-Nagel) in accordance with the protocol provided by the manufacturer. The RNA samples were subsequently treated with RNase-free DNase (catalogue no. AM2222; Ambion) and purified with a NucleoSpin RNA cleanup kit (catalogue no. 740948.50; Macherey-Nagel). Two micrograms of each RNA samples was used as a template for cDNA synthesis with random primers, using the ThermoScript reverse transcription-PCR (RT-PCR) system (catalogue no. 11146-016; Invitrogen). The program for cDNA synthesis was 25°C for 10 min, followed by 50 min at 50°C. Ten percent of the cDNA synthesis reaction mixture (2 μl) was used as a template for each subsequent PCR, using the following gene-specific primer pairs: RT-hrdB1/RT-hrdB2, RT-scbA1/RT-scbA2, RT-scbR1/RT-scbR2, and RT-cpkO1/RT-cpkO2 (Table 6). The PCR program used was as follows: (i) 95°C for 5 min, (ii) 95°C for 40 s, (iii) 62°C for 40 s, (iv) 72°C for 40 s, and (v) 72°C for 10 min, with the second to fourth steps repeated for 35 cycles. Negative controls were carried out without the addition of reverse transcriptase in order to demonstrate that amplified products were derived from RNA transcripts and not from chromosomal DNA.

TABLE 3. Quantitative analysis of undecylprodigiosin

Strain	Concn (μmol/g [dry wt] cells) (R%)[a]			
	pWHM3	pWHM3-*SCO3201*	WT	Δ*SCO3201*
S. coelicolor M145	0.39 ± 0.012	0.25 ± 0.010 (36)	0.37 ± 0.038	0.30 ± 0.004
S. lividans TK24	0.09 ± 0.005	0.09 ± 0.002	0.08 ± 0.002	0.09 ± 0.015
S. lividans TK24/Δ*ppk*::Ω*hyg*	0.57 ± 0.050	0.19 ± 0.037 (67)	0.65 ± 0.058	0.55 ± 0.030

[a] R%, percentage of reduction of RED or ACT production; WT, wild type.

TABLE 4. Quantitative analysis of actinorhodin

Strain	Concn (μmol/g [dry wt] cells)[a]			
	pWHM3	pWHM3-*SCO3201* (R%)	WT	Δ*SCO3201*
S. coelicolor M145	50.25 ± 2.26	13.36 ± 0.81 (74%)	53.43 ± 3.66	41.49 ± 1.81
S. lividans TK24	ND	ND	ND	ND
S. lividans TK24/Δ*ppk*::Ω*hyg*	24.16 ± 1.60	1.18 ± 0.03 (95%)	21.51 ± 2.97	19.47 ± 1.13

[a] Values shown represent the sum of the intracellular and extracellular values. R%, percentage of reduction of RED or ACT production; WT, wild type; ND, not detected.

RESULTS

The cloning of $SCO3201_{SL}$ on a multicopy plasmid strongly inhibits ACT biosynthesis in *S. coelicolor*. *S. coelicolor* produces copious amount of the blue-pigmented antibiotic actinorhodin (ACT), whereas *S. lividans* hardly produces any detectable amount of this compound. Since it is now known from the comparison of the *S. coelicolor* (http://www.sanger.ac.uk/) and *S. lividans* (http://www.broadinstitute.org/) genome sequences that approximately 600 genes that are present in the *S. lividans* genome are absent in the *S. coelicolor* genome and vice versa, we hypothesized that the very different abilities of these two strains to produce antibiotics might be due to the presence in the *S. lividans* genome of a major negative regulator of ACT production that would be absent or not functional in *S. coelicolor*. In an attempt to clone this putative negative regulator, a genomic library of *S. lividans* DNA was constructed in the high-copy-number replicative plasmid pIJ702 (33). Transformation of *S. coelicolor* M145 with 10 μg of this library yielded approximately 4,000 transformants. The average size of genomic DNA inserts being approximately 4 kb, the library coverage was the equivalent of two genomes. An unique colorless transformant (ACT^-) was obtained. The ACT^- phenotype was confirmed upon retransformation of *S. coelicolor* with the plasmid extracted from this unique white transformant. Sequence analysis of the insert carried by the plasmid revealed that the plasmid contains two complete genes homologous to *SCO3201* and *SCO3200* of *S. coelicolor* (Fig. 1). These two converging genes encode regulators of the TetR and DeoR families, respectively. In order to determine which of these two genes was responsible for the ACT^- phenotype, each gene and its upstream region as well as the upstream regions alone were cloned separately on the multicopy plasmid pIJ702 (33) and introduced into *S. coelicolor*. These experiments clearly demonstrated that only the overexpression of *SCO3201* of *S. lividans* ($SCO3201_{SL}$), encoding a regulator of the TetR family, was responsible for the ACT^- phenotype.

The overexpression of $SCO3201_{SC}$ also strongly inhibits ACT biosynthesis of *S. coelicolor*. In order to determine whether the equivalent gene of *S. coelicolor* was or was not functional, *SCO3201* of *S. coelicolor* ($SCO3201_{SC}$) and $SCO3201_{SL}$ were both cloned in the high-copy-number plasmid pWHM3 under the control of the strong constitutive *ermE* promoter. These constructs were introduced into *S. coelicolor* M145, and indeed, both conferred the ACT^- phenotype (Fig. 2), indicating that $SCO3201_{SC}$ was functional despite the 7 nucleotide changes leading to 3 amino acid changes (besides the start codon) compared with $SCO3201_{SL}$ (http://www.broadinstitute.org/) (see Fig. S1 in the supplemental material). These results invalidated our hypothesis demonstrating that *SCO3201* obviously does not encode the hypothetical negative regulator of ACT biosynthesis that we were looking for.

The overexpression of $SCO3201_{SL/SC}$ inhibits both metabolic and morphological differentiation of *S. coelicolor*. In order to assess the effect of the overexpression of $SCO3201_{SL/SC}$ on the global ability of *S. coelicolor* M145 as well as of the *ppk* mutant of *S. lividans* TK24 to overproduce antibiotics, these strains as well as *S. lividans* TK24 were transformed either by the plasmid pWHM3-$SCO3201^{SC}$ or pWHM3-$SCO3201^{SL}$ or by pWHM3-*ermE* (empty vector as a control). The presence of any of the pWHM3-based multicopy plasmids did not change the growth pattern of the studied strains that were grown on the R2YE solid medium for 84 h (data not shown). ACT, RED, and CDA were assayed with these cultures as described in Materials and Methods. The results shown in Table 4 indicate that the introduction of pWHM3-$SCO3201^{SL/SC}$ led to 74% and 95% reductions of ACT production of *S. coelicolor* M145/pWHM3 and of *S. lividans* TK24/Δ*ppk*::Ω*hyg*/pWHM3, respectively. Similarly, the results shown in Table 3 indicate that the introduction of pWHM3-$SCO3201^{SL/SC}$ led to 36% and 67% reductions of RED production of *S. coelicolor* M145/pWHM3 and of *S. lividans* TK24/Δ*ppk*::Ω*hyg*/pWHM3, respectively. Regarding CDA, the diameters of the growth inhibition zones of *M. luteus* attributed to CDA production around patches of *S. coelicolor* M145 were approximately 1.5-fold larger than those seen around patches of the two *S. lividans* strains (Table 2; see also Fig. S3 in the supplemental material), indicating that *S. coeli-*

TABLE 5. Quantitative analysis of actinorhodin (intracellular and extracellular values)

Strain	Concn (μmol/g [dry wt] cells)[a]					
	pWHM3			pWHM3-*SCO3201*		
	In	Out	Ratio (out/in)	In	Out	Ratio (out/in)
S. coelicolor M145	7.13 ± 0.34	43.12 ± 2.07	5.52	2.92 ± 0.82	10.44 ± 0.48	3.92
S. lividans TK24/Δ*ppk*::Ω*hyg*	1.87 ± 0.20	22.28 ± 1.20	11.91	0.64 ± 0.05	0.54 ± 0.03	0.84

[a] In, intracellular; Out, extracellular.

TABLE 6. Synthetic oligonucleotides used in this study

Primer	5′ → 3′ sequence[a]	Position(s)	Purpose
PtetR1-XbaI	CAA**TCTAGA**TTCGGCAGGCTATGCCGCTG	−394 to −1	Amplification of the *SCO3201* promoter region for promoter probing (*xylE* fusion)
PtetR1-HindIII	CAA**AAGCTT**CGGATGCCGCGTGACGTG		
PtetR2	CACTTCGGCAGGCTATGCC	−240 to +3	Amplification of the *SCO3201* promoter region for EMSA
PtetR2′	ACCGAAGACCACGTCCCGAC		
UtetR-BamHI	CG**GGATCC**CGCAAGGGCTTCAAGTTCTC	−999 to −1	Amplification of the 1-kb fragment located upstream of *SCO3201*
UtetR-XbaI	GC**TCTAGA**TTCGGCAGGCTATGCCGCTG		
DtetR-HindIII	CCAAGCTTGGCACGCGGTCGAGGGCCTG	+754 to +1711	Amplification of the 1-kb fragment located downstream of *SCO3201*
DtetR-XbaI	GCTCTAGAGGTCGCTCCGTCGGGGGTGA		
ItetR1	ACCGAAGACCACGTCCCGAC	−240	Verification of the interruption of *SCO3201*
ItetR2	CGGATCTCGGCCCAGTTGAC		
GSP1	CTCTCGGCCAGACGGATGAG	+416	Gene-specific primer (GSP) for 5′-RACE PCR
GSP2	GACGGCCTCTTCCTTGGTGGCTGG	+240	
GSP3	CGATGTCCTCGGCC	+179	
ExpTetR1-NdeI	CAA**CATATG**GTGAGCAGCACCATTCCAGCACTTC	+1 to +708	Amplification of *SCO3201* for protein expression
ExpTetR2-XhoI	CAA**CTCGAG**CCCCTCTTCCGCGGGCCC		
RT-hrdB1	AAGGAAGACGGCGAGCTTCT	+481 to +1060	RT-PCR
RT-hrdB2	GCACCGGGATACGGATGGTG		
RT-scbA1	GATCAATTCTGCGTCCGATG	+68 to +557	RT-PCR
RT-scbA2	GTAGACTTGAGGACTGGTG		
RT-scbR1	CAAGCAGGACCGGGCGATC	+130 to +664	RT-PCR
RT-scbR2	CTTCTGCAGCAGCGCGTAGC		
RT-cpkO1	GTCCACTCGAGGTGTTGTCC	+55 to +546	RT-PCR
RT-cpkO2	GGTAGTCCTCCAGGACATCG		
Plabel	AGCCAGTGGCGATAAG		Cy5 labeling
PscbA1	AGCCAGTGGCGATAAGCCAGGAATCATGTGATGCCG	−230 to +38	Amplification of the *scbA* promoter region for EMSA
PscbA2	AGCCAGTGGCGATAAGCCTTGGACTGGAAGTGGAAG		
PscbR1	AGCCAGTGGCGATAAGGGCCATCAGGAAGTGGTAGC	−303 to +10	Amplification of the *scbR* promoter region for EMSA
PscbR2	AGCCAGTGGCGATAAGACCCATGCCCGAAGCAGTAG		
PcpkO1	AGCCAGTGGCGATAAGCATCCGGGACACCGACGGAG	−315 to +67	Amplification of the *cpkO* promoter region for EMSA
PcpkO2	AGCCAGTGGCGATAAGCACCTCGAGTGGACCGAGC		

[a] Engineered restriction enzyme sites are in bold. Underlined nucleotides show no homology to the template; they were used for Cy5 labeling.

color was a stronger producer of CDA than *S. lividans* (wild type and *ppk* mutant). The introduction of pWHM3-*SCO3201*$^{SL/SC}$ in these strains led to a total absence of the growth inhibition zone of *M. luteus*, suggesting that the overexpression of *SCO3201*$_{SC/SL}$ led to a strong repression of CDA production.

Furthermore, since we noticed that aerial mycelium formation and sporulation were delayed in *S. coelicolor* M145/pWHM3-*SCO3201*$^{SC/SL}$ grown on R2YE, we tested the sporulation ability of this strain on SFM medium (20), a medium known to promote good sporulation. Indeed, we confirmed that the introduction of this plasmid into *S. coelicolor* M145 strongly inhibited the morphological differentiation process and sporulation in this strain (Fig. 2). Curiously, the introduction of this plasmid into *S. lividans* TK24, the wt (Fig. 2), and the *ppk* mutant (data not shown) did not lead to an obvious

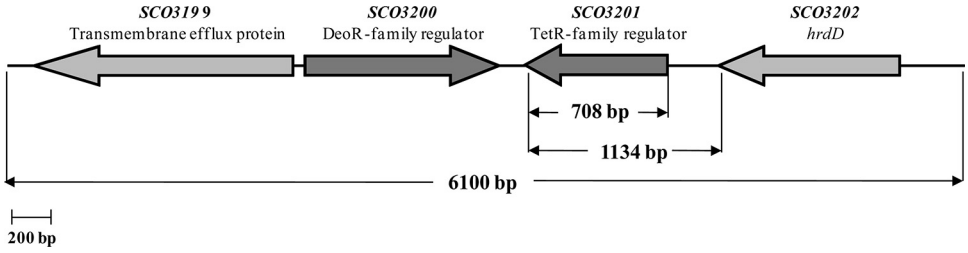

FIG. 1. Schematic representation of the genetic surroundings of *SCO3201*.

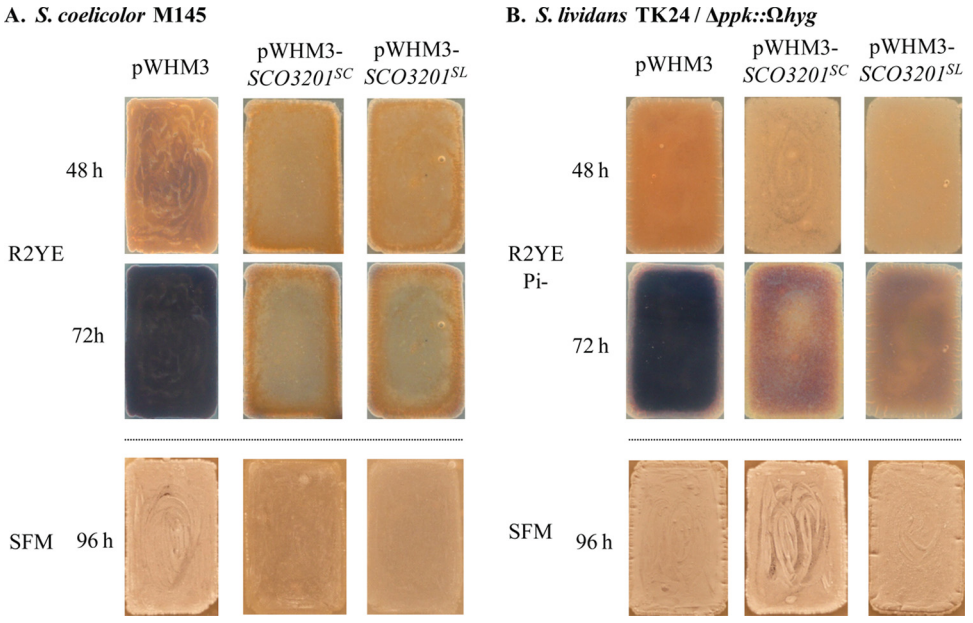

FIG. 2. Phenotypes resulting from *SCO3201* overexpression. Effects of the introduction of pWHM3, pWHM3-$SCO3201^{SC}$, and pWHM3-$SCO3201^{SL}$ in *S. coelicolor* M145 (A) and *S. lividans* TK24/*ppk*::Ω*hyg* (B) on ACT production (lawns grown on R2YE medium for 48 and 72 h) and on sporulation (lawns grown on SFM medium for 96 h).

inhibition of the morphological differentiation process (sporulation) of these strains. These observations confirm that the regulatory circuits controlling morphological and metabolic differentiation in these two closely related species are somehow different.

The interruption of *SCO3201* did not correlate with any obvious phenotype. Since the overexpression of *SCO3201* had a strong negative effect on the antibiotic production of *S. coelicolor* M145 as well as of the *ppk* mutant strain of *S. lividans* TK24, we thought that conversely, its interruption might correlate with an enhanced level of antibiotic production, at least in *S. lividans* TK24. In order to test this hypothesis, this gene was interrupted in *S. lividans* TK24 (wt and *ppk* mutant) as well as in *S. coelicolor* M145 as described in Materials and Methods. The interruption of *SCO3201* did not lead to the expected phenotype of ACT overproduction in *S. lividans* TK24 or to any other obvious phenotype in that strain, in the *ppk* mutant, or in *S. coelicolor* M145 (Tables 2 to 5). This lack of an obvious phenotype again confirmed that this gene was not encoding the master negative regulator of ACT biosynthesis that we sought. However, a slightly more precocious sporulation of the *SCO3201* knockout mutant of *S. coelicolor* M145 could be noticed on SFM medium at 48 h (data not shown). These unexpected negative results suggest that under the condition of overexpression, this TetR regulator might interact with the promoter regions of the target genes of some of the 151 TetR regulators present in the *S. coelicolor* genome (51), those that are most likely to play a role in the regulation of the metabolic and morphological differentiation process of that bacteria (37, 56, 60, 61). In order to test this hypothesis, and considering that this "experimental artifact" could prove to be quite useful in elucidating the regulatory circuits governing metabolic and physiological differentiation in *Streptomyces*, the study of this regulatory gene was pursued.

***In vitro*, SCO3201 interacts with its own promoter region.** Since TetR regulators often negatively regulate their own expression by binding, as dimers, to operator sites located in their promoter region (3, 12, 31, 53), the ability of $SCO3201_{SC}$ to interact with its own promoter region was tested with the EMSA technique using a biotin-labeled *SCO3201* promoter region (fragment *SCO3201*p) and His_6-$SCO3201_{SC}$ purified from *E. coli*. The result shown in Fig. 3A clearly demonstrated that purified His_6-$SCO3201_{SC}$ was indeed able to specifically bind to and delay its own promoter region since two shifted bands were detected. The more abundant shifted band, band II, might correspond to the binding of a dimer of $SCO3201_{SC}$, whereas the much less abundant shifted band, band I, whose amount increases with increasing amounts of $SCO3201_{SC}$ (Fig. 3A, lanes 2 to 5), might correspond to the binding of more than one dimer to secondary operator sites present in the promoter region or to a tetramer to the core binding site (Fig. 4). The addition of an excess amount of an unlabeled *SCO3201* promoter fragment to the reaction mixture indeed led to the disappearance of the shifted bands (Fig. 3A, lanes 6 and 7), demonstrating the specificity of the $SCO3201_{SC}$-*SCO3201* promoter interaction. A biotinylated DNA fragment internal to the $SCO3201_{SC}$ coding sequence (positions +4 to +250) was also used as a negative control in the EMSA, and indeed, no significant shifting of this fragment was observed in the presence of $SCO3201_{SC}$ (data not shown). Similar results were obtained with purified His_6-$SCO3201_{SL}$ (data not shown), indicating that both proteins possess the ability to interact with the *SCO3201* promoter region that is identical in *S. coelicolor* and *S. lividans*.

When similar EMSA experiments were carried out with crude protein extracts (Fig. 3B) originating from the wild-type strain of *S. lividans* grown on solid R2YE, two clearly delayed discrete bands were seen besides a very abundant but less

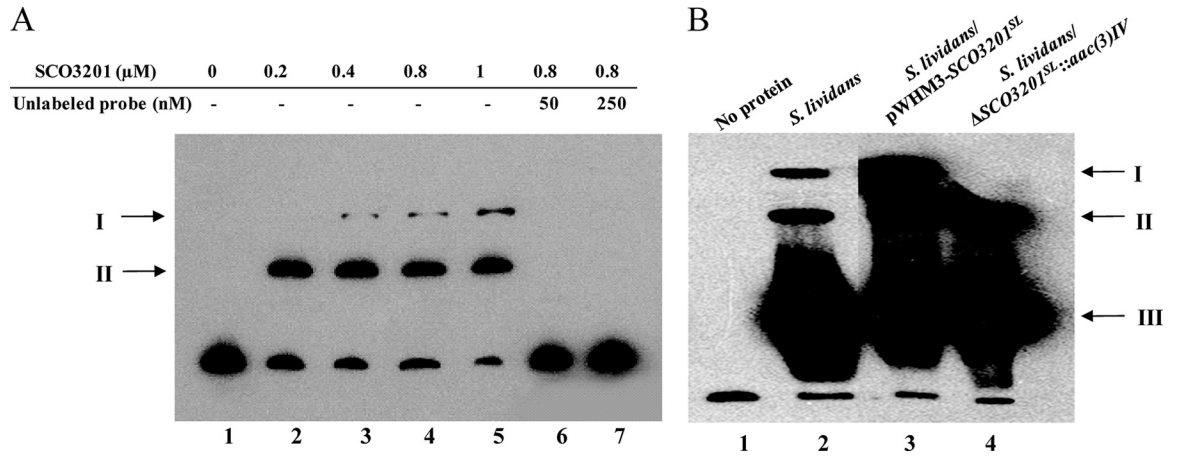

FIG. 3. Electrophoretic mobility shift assays of the *SCO3201* promoter region with purified His_6-$SCO3201_{SC/SL}$ (A) and with cell crude extracts (B) from *S. lividans* TK24 (WT), *S. lividans* TK24/pWHM3-$SCO3201^{SL}$, and *S. lividans* TK24/*SCO3201*::*aac(3)IV*. In both cases, 0.1 pmol of biotin-labeled *SCO3201* promoter was used. The shifted bands are indicated by arrows.

delayed band. When crude extracts of the *SCO3201* mutant of that strain were used, the topmost delayed band disappeared, whereas it was reinforced when crude extracts were prepared from the strain overexpressing $SCO3201_{SL}$. These results clearly demonstrated that $SCO3201_{SL}$ was responsible for the retardation of the most delayed band but that another transcriptional regulator might be responsible for the retardation of the second most delayed band. The less delayed but very intense band is similarly abundant with all crude extracts, suggesting that it might be due to the interaction of the *SCO3201* promoter region with the RNA polymerase.

The core of the SCO3201 binding site overlaps with the −35 box of its promoter region. In order to position the SCO3201 binding site relative to the promoter region, the transcriptional start site of *SCO3201* was determined using 5′-RACE PCR as described in Materials and Methods. The results (Fig. 4; see also Fig. S2A in the supplemental material) indicated that the transcriptional start site coincides with the first G of the putative GTG translation initiation codon of *SCO3201*, which is frequently used in *Streptomyces*. *SCO3201* is thus transcribed as a leaderless mRNA, like many other *Streptomyces* genes (24, 52). The determination of the transcription start point of *SCO3201* led to the proposal of the putative −10 and −35 promoter sequences boxed in Fig. 4. These promoter sequences show similarity to the vegetative promoters recognized by $E\sigma^{70}$ of *E. coli* and have a classical spacing of 17 nucleotides (nt) (57).

In order to determine the $SCO3201_{SC}$ binding site in the *SCO3201* promoter region, DNase I footprinting assays of the fluorescently labeled *SCO3201* promoter region were performed in the presence and absence of $SCO3201_{SC}$ and analyzed with a capillary DNA sequencer, as described in Materials and Methods. The results showed three closely located intermittently protected regions, extending from position −13 to position −56 relative to the transcription start point (a total of 44 bp) on both the coding and the complementary strands (see Fig. S2B in the supplemental material). Full protection was achieved at a very low His_6-$SCO3201_{SC}$ concentration (0.02 μM) in each case. The central SCO3201 binding site (BS^C) encompasses an 11-nt palindromic sequence (TGGCA GATTCTGCCA) that overlaps the −35 sequence of the promoter region (Fig. 4). A second protected site (BS^R), located to the right of BS^C, encompasses the left part of a 5-nt inverted repeat (CGGCA), located 12 nt upstream of the transcriptional start site. This short palindromic sequence has four nucleotides (GGCA) in common with BS^C. The third protected site (BS^L) is located on the left of BS^C and encompasses the 10-nt very-GC-rich palindromic sequence (ACCCCGGG GT). The three nucleotides at each extremity of this short palindromic sequence are the same as those of BS^C (but in the opposite orientation). The footprint analysis confirmed the direct interaction of SCO3201 with its own promoter region, indicating that SCO3201 was negatively regulating its own transcription by blocking the access of the RNA polymerase to its promoter region.

The negative autoregulation of SCO3201 was confirmed, *in vivo*. In order to confirm, *in vivo*, the negative autoregulation of *SCO3201* expression, a transcriptional fusion of the promoter

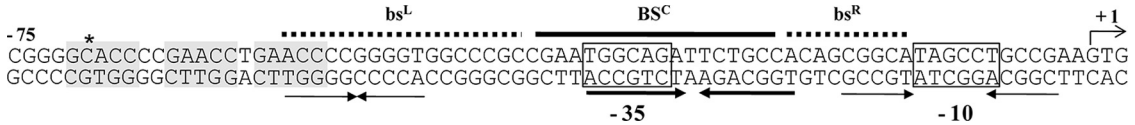

FIG. 4. Sequence of the SCO3201 promoter region. The transcription start point is indicated by a bent arrow. The translation start codon GTG is shown in boldface letters. The putative −10 and −35 boxes are boxed. The DNA region protected by SCO3201 from DNase I digestion is underlined by continuous (core region; BS^C) or dotted (secondary regions; BS^R and BS^L [here shown in lowercase to correspond to their being minor binding sites relative to BS^C]) lines. The core SCO3201 binding site (BS^C) overlaps the −35 sequence. Palindromes are indicated by inverted arrows. The three well-conserved direct repeats located upstream of the −35 box are shaded (a mismatched nucleotide is marked with an asterisk).

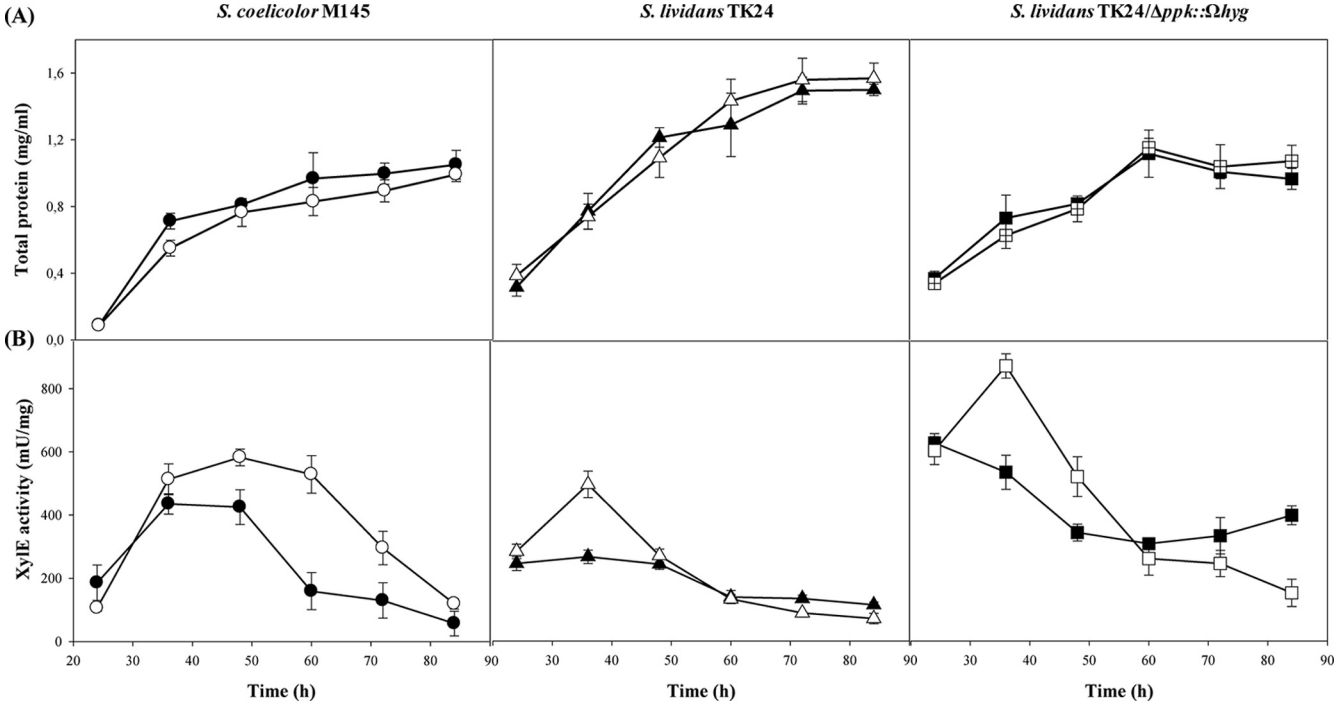

FIG. 5. (A) Growth curves of *S. coelicolor* M145 (circle), *S. lividans* TK24 (triangle), *S. lividans* TK24/*ppk*::*hyg* (square), and their corresponding *SCO3201* knockout mutants. (B) XylE activities resulting from the expression of the *xylE* reporter gene put under the control of the *SCO3201* promoter in *S. coelicolor* M145, *S. lividans* TK24, *S. lividans* TK24/*ppk*::*hyg*, and their corresponding *SCO3201* knockout mutants grown in liquid R2YE medium. Filled and open symbols represent parental and *SCO3201* mutant strains, respectively.

region of $SCO3201_{SC}$ (which shows 100% identity in nucleotide sequence to that of $SCO3201_{SL}$) with the *xylE* reporter gene was constructed using the high-copy-number promoter probe vector pIJ4083 (33). This plasmid was introduced into the wt and *ppk* mutant strains of *S. lividans* TK24, into *S. coelicolor* M145, and into their corresponding *SCO3201* knockout mutants. The XylE activities were assayed for these strains grown in liquid R2YE medium as described in Materials and Methods, and the quantitative results are shown in Fig. 5. The expression profiles of the *SCO3201*p-*xylE* fusion were quite different in the three strains. However, in all strains, the expression level of the *xylE* fusion was higher during active growth (<50 h) than in stationary phase (>60 h) and was higher in the antibiotic-producing strains (and higher in *S. coelicolor* than in the *ppk* mutant of *S. lividans*) than in the non-antibiotic-producing strain (*S. lividans*). In the three strains, the interruption of *SCO3201* led to a clear increase of the *xylE* fusion expression, demonstrating the negative autoregulation of SCO3201. However, whereas the expression of the *xylE* fusion continued during stationary phase in the *SCO3201* mutant of *S. coelicolor*, the expression of this gene in the *SCO3201* mutants of the wt and the *ppk* mutant of *S. lividans* was rather transitory and centered on the 36-h point. These observations suggest that the regulatory circuits governing *SCO3201* expression are quite different in these two species.

SCO3201 directly interacts with the *scbA* promoter region *in vitro* and regulates its transcription *in vivo*. Sequences similar to the SCO3201 operator site were searched in the promoter regions of other known *Streptomyces* regulators of the TetR family using the MEME algorithm (multiple expectation maximization [EM] for motif elicitation) (2). The best hit found was in the promoter region of *scbA*, a gene encoding a protein required for γ-butyrolactone SCB1 biosynthesis (61). The expression of *scbA* is known to be positively regulated by the divergently located TetR-like regulator ScbR through direct interaction (45, 61). ScbR was also shown to negatively regulate its own expression and that of *cpkO*, a gene encoding the putative pathway-specific transcriptional regulator of the cryptic type I polyketide gene cluster, also through direct interaction (63). The ability of SCO3201 to shift the promoter regions of *scbA*, *scbR*, and *cpkO* was then tested. Only the promoter region of *scbA* was shifted by the addition of histidine-tagged SCO3201 (Fig. 6A). The shifting was abolished by the addition, in large excess, of the unlabeled probe (Fig. 6A, lane 6) demonstrating the specificity of the protein-DNA interaction. With the MEME software program, the *scbA* promoter region was found to share the highest level of similarity with the SCO3201 binding site since a region of 26 nt, with 17 identical nucleotides, was overlapping with most of BS^C and BS^R (Fig. 6C), whereas the ScbR and *cpkO* promoter regions shared a shorter region of similarities, encompassing mostly BS^L and/or the left part of BS^C (see Fig. S4 in the supplemental material). SCO3201 is thus predicted to have less affinity for the *scbR* and *cpkO* promoter regions than for the *scbA* promoter region.

In order to determine whether SCO3201 was or was not interfering with the ScbR/ScbA/CpkO regulatory circuit *in vivo*, the levels of transcription of *scbA*, *scbR*, and *cpkO* were analyzed by RT-PCR in *S. coelicolor*/pWHM3 and *S. coelicolor*/pWHM3-*SCO3201* at 24 h and 48 h. As shown in Fig. 6B, the

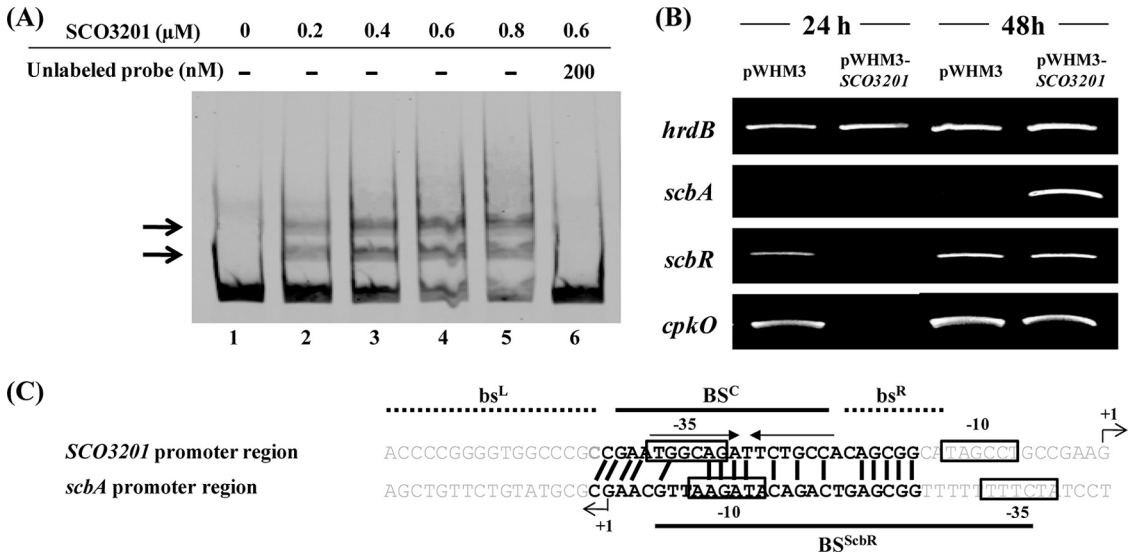

FIG. 6. (A) Electrophoretic mobility shift assay of the *scbA* promoter region with purified His_6-SCO3201. Cy5-labeled DNA probe (0.1 pmol) was used for each reaction. The shifted bands are indicated by arrows. (B) Assessment of *scbA*, *scbR*, and *cpkO* transcript levels by RT-PCR in *S. coelicolor*/pWHM3 and *S. coelicolor*/pWHM3-*SCO3201*^{SC} at 24 and 48 hours. (C) Alignment of the promoter regions of *SCO3201* and *scbA*. Sites of banding of SCO3201 to its native promoter and of ScbR to the *scbA* promoter are over- and underscored, respectively. The palindromic sequence constituting most of the core binding site (BS^C) of *SCO3201* is indicated by inverted arrows. Putative −10 and −35 regions are boxed. Transcriptional start points are indicated by bent arrows. The region highly conserved between the two promoter regions is in bold, and the identical nucleotides are marked by bars.

overexpression of *SCO3201* correlates with a downregulation of *scbR* and *cpkO* expression at 24 h but not at 48 h and with the upregulation of *scbA* expression at both 24 h and 48 h. The relief of SCO3201-mediated repression of *scbR* and *cpkO* expression at 48 h might be related to the weak affinity of SCO3201 for its target sites in the *scbR* and *cpkO* promoter region. This weak affinity might be further destabilized by the presence of the SCO3201 ligand at late time points (48 h).

Overexpression of SCO3201 reduces the efficiency of ACT export, at least in *S. lividans*. In Table 5, the ratios between extracellular (exported) and intracellular ACT were calculated for the antibiotic-producing *S. coelicolor* M145 and *S. lividans* Δ*ppk*::Ω*hyg* strains containing either pWHM3 or pWHM3-*SCO3201*. This ratio was found to be 14-fold (11.91/0.84) lower in the *S. lividans* Δ*ppk*::Ω*hyg* strain containing pWHM3-*SCO3201* than in that strain containing empty pWHM3 but only 1.4-fold (5.52/3.92) lower in the equivalent *S. coelicolor* strains. This observation suggests a possible involvement of SCO3201 in the regulation of the expression of the genes encoding the ACT export machinery, at least in *S. lividans*. Indeed, the ACT export machinery, encoded by the *actAB* operon, is under the control of the TetR regulator *act*II-ORF1 (*actR*) (14, 59, 60), and SCO3201 might be able to control, *in vivo*, the expression of this operon. The strikingly differential effects of SCO3201 overexpression in the two species on the export of ACT is not understood but might be related to differences in the regulatory circuits governing ACT export in the two species or to the greater abundance in *S. coelicolor* than in the *ppk* mutant of *S. lividans* of ligands able to dissociate SCO3201 from its binding sites.

DISCUSSION

The present paper describes the characterization of a novel regulator of the TetR-family, SCO3201, one among the 151 TetR regulators present in the *S. coelicolor* genome (51), whose overexpression was shown to have a strong negative effect on the metabolic (antibiotic production) and morphological (sporulation) differentiation process of *S. coelicolor* M145. The overexpression of *SCO3201* led to an apparent total shutoff of CDA biosynthesis in the three strains tested, to a strong reduction of ACT biosynthesis, and to a more moderate reduction of RED biosynthesis. The reduction of ACT and RED biosynthesis was always more drastic in the *ppk* mutant strain of *S. lividans* TK24 than in *S. coelicolor* M145. These differences might indicate the existence of different regulatory circuits controlling antibiotic production in these two species or a greater abundance in *S. coelicolor* than in *S. lividans* of the ligand able to dissociate SCO3201 from its target sites. Furthermore, these observations confirm the existence of common regulatory circuits governing the biosynthesis of secondary metabolites that require different types of precursors for their biosynthesis (amino acids versus acetyl-coenzyme A [CoA] derivatives).

Interestingly, the overexpression of *SCO3201* had also a negative effect on the morphological differentiation process of *S. coelicolor* M145 but not of *S. lividans* TK24 (wt and *ppk* mutant). These observations demonstrate that the regulatory circuits controlling the morphological differentiation process are somehow different in these two closely related species. Despite the overexpression of *SCO3201*, *S. lividans* sporulates, indicating either that some of the SCO3201 operator sites are missing in front of key genes involved in the positive regulation

of sporulation of that strain or that some negative regulatory elements of the sporulation process are missing in *S. lividans*. Indeed, it was demonstrated that some genomic islands larger than 25 kb and comprising more than 600 genes were present in the *S. coelicolor* genome but absent in the *S. lividans* genome (30).

The SCO3201 regulator was shown to negatively autoregulate its own synthesis, and its operator site in its own promoter region was identified as the 15-bp palindromic sequence (TG GCAGATTCTGCCA) that is a typical binding site for homodimer transcriptional regulators of the TetR family (51). This binding site overlaps with the −35 promoter sequence, impairing RNA polymerase binding. Footprinting experiments revealed that the binding of SCO3201 wobbles between its core binding site and two lateral sequences with reduced sequence conservation with the core sequence and imperfect symmetry (Fig. 4). The binding of TetR regulators to their target sites is usually relieved by small inducer molecules that trigger drastic conformational changes (22). In the case of SCO3201, the "inducer molecule" is not known, but it is interesting to note that the C terminus of this regulator, which usually includes the ligand binding motif in other TetR regulators, bears some weak similarities with leucine/isoleucine/valine-binding protein (LIVBP), suggesting that some branched amino acids (Leu, Ile, and Val) might be able to dissociate SCO3201 from its target sites. However, in the multicopy situation under the condition of overexpression, the TetR ligands might not be abundant enough to relieve the SCO3201-mediated repression of gene expression.

Furthermore, our band-shifting experiments with the *SCO3201*promoter region carried out in the presence of crude extracts of the different strains (Fig. 3B) indicated that the regulation of *SCO3201* expression might involve another regulator besides SCO3201. Indeed, in the *SCO3201* mutant of the *S. lividans*, the expression of the *xylE* reporter gene under the control of the *SCO3201* promoter was shown to be transitory (peak at 36 h), suggesting the contribution of another positive or negative regulator besides SCO3201 mediating transitory activation or repression relief, respectively. The time point of 36 h usually corresponds to the transition between a first phase of active growth and a second phase of slower growth that precedes the entry into stationary phase. If a second negative regulator controls *SCO3201* expression, it might be another TetR-like regulator recognizing the SCO3201 operator sequence. Alternatively, we cannot exclude that the second putative regulator is a positive one, since three rather well conserved direct repeats of the sequence GAACC were found 36 bp upstream of the −35 sequence, the usual position of activator sites (Fig. 4). In the two antibiotic-producing strains (the *S. coelicolor* strain and the *ppk* mutant of *S. lividans*), the expression of *SCO3201* is higher than in the *S. lividans* strain, suggesting that either repression of *SCO3201* expression by both SCO3201 and the other putative negative regulator is partially relieved or that activation by an unknown regulator takes place concomitantly to the relief of the SCO3201-dependent repression.

However, the most surprising outcome of this study is that whereas the overexpression of *SCO3201* leads to a very strong phenotype, the interruption of this gene did not correlate with any obvious phenotype in any of the three studied strains. This indicated that the strong phenotypic consequences of the overexpression of *SCO3201* were somehow an "experimental artifact" linked to SCO3201 abundance. Under conditions of great SCO3201 abundance, the latter might be able to interact with the operator sequences of other TetR regulators not closely related to its own operator site. Indeed, other TetR regulators, including ScbR, CprA, CprB, RrdA, SCO1712, and ActR, were shown to be involved in the regulation of antibiotic production or antibiotic export (37, 48, 49, 59, 61). We demonstrated that SCO3201 was able to mimic ScbR activity, enhancing *scbA* expression *in vivo*, indicating that this "experimental artifact" could prove quite useful in deciphering the regulatory circuits controlling the metabolic and morphological differentiation in *S. coelicolor* and *S. lividans*. Indeed, the deletion of *scbA* was previously shown to lead to an increase in ACT and RED biosynthesis (61), so its overexpression is likely to contribute to the observed reductions of ACT and RED biosyntheses in *S. coelicolor* overexpressing *SCO3201*. Other targets of SCO3201 remain to be characterized. This characterization is in process by *in silico* as well as by *in vivo* and *in vitro* capture of genomic DNA-SCO3201 cross-link approaches.

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